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NOVEL MEMBRANE-SECRETED PROTEIN

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Technical field

This invention pertains to novel membrane-secreted proteins participating in the differentiation of bone cells, the DNA encoding the proteins, the vector containing the DNA, the host cells that sustain the vector, the antibody against the proteins, the screening method for chemicals using the proteins, and to the chemicals which can be isolated by the screening method.

Technical background

The phenomenon of bone regeneration due to the action of osteoblasts, that is, bone formation, is an important phenomenon for vertebrates, in order to sustain life. As the already known factors concerning bone formation, there are estrogen, calcitonin and parathyroid hormone (PTH) of the hormone groups, bone morphogenic protein (BMP) as the proliferation factor, and, additionally, drugs such as activated vitamin D, calcium preparations, vitamin K2, etc. Among them, estrogen, calcitonin, activated vitamin D and calcium preparations are currently being utilized as treatment drugs for improving symptoms, such as those of osteoporosis. However, each of these drugs is used for preventing loss of bone mass involving the mechanism of resorption of the bone rather than for increasing the bone mass, and the current situation is that there has not been an effective drug developed for promoting bone formation.

On the other hand, BMP, which commands certain expectations as a novel treatment drug for various bone-related diseases, is the only cytokine which is a heterotopic formation signal. BMP is considered useful in bone formation (endochondral ossification) which occurs during the healing reaction when there is an actual bone fracture or injury, that is, occurring when cartilaginous callus is being replaced by new bone (Duprez DM, Coltey M, Amthor H, Brickell PM, Tickle C (1996), Dev Biol 174:448-452. Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures, Nakase T, Nomura S, Yoshikawa H, Hashimoto J, Hirota S, Kitamura Y, Oikawa S, Ono K, Takaoka K (1994), J Bone Miner Res 9:651-659. Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing).

However, there has been no report of concrete confirmation of actual participation of BMP in the bone formation which accompanies the resorption of the bone during the normal course of bone formation. Therefore, it is hard to say that BMP could be used as a drug for promoting bone formation, which relies on its promoting and activating action for the differentiation of osteoblasts, which is necessary in the normal course of bone formation. That is, the fact is that, currently, there have been no reports on the factors concerning the normal course of bone formation.

Presentation of the invention

The objective of this invention lies in providing novel proteins, and their genes, which participate in the normal course of bone formation. Still another objective of the present invention lies in providing the vector inserting the genes, the host cells sustaining the vector, and the antibody binding to the proteins. Furthermore, still another objective of the present invention lies in providing a screening method using the proteins for chemicals which bind to the proteins as ligands.

As a result of vigorous studies aimed at resolving the aforementioned objectives, the present inventors succeeded in isolating genes encoding 3 kinds of membrane-secreted proteins from osteoblast-like cell lines, using a cloning method specific for genes encoding membrane-secreted proteins. By analyzing one of the genes, the present inventors discovered that the protein encoded by the gene is a novel receptor gene which has only the extracellular region and binds to cellular membrane via a GPI anchor and carries therein a cysteine-rich region conserved in the TNF receptor superfamily. The present inventors also discovered that, when the protein is highly expressed in an osteoblast-like cell line, the proliferation of the cells are suppressed, the cells are morphologically changed, and the alkalinene phosphatase activity, which is one of the indicators for the differentiation of osteoblasts, is elevated. Furthermore, from the fact that the proteins so isolated are participating in the differentiation of osteoblasts, it was discovered that the proteins could be utilized to screen prospective drug chemicals for bone-related diseases.

That is, the present invention pertains to novel membrane-secreted proteins and the genes, and the screening method for prospective drug chemicals using the proteins, and more particularly, it pertains to

(1) proteins formed by the amino acid sequence shown in sequence No.: 1 or 2, and those having the amino acid sequences resulting from substituting, deleting or additions of one or more amino acids in the amino acid sequences of the proteins, and these proteins having induction activity on the differentiation of bone cells,

(2) proteins encoded by DNA hybridizing with the DNA having the base sequence of sequence No.: 3, and the proteins having induction activity on the differentiation of bone cells,

(3) DNAs encoding the proteins described in (1) and (2),

(4) vectors inserted with the DNA described in (3),

(5) host cells sustaining the vectors described in (4),

(6) antibodies binding to the proteins described in (1) and (2),

(7) a screening method for chemicals binding to the proteins described in (1) and (2), with the method consisting of

(a) a process of bringing test samples into contact with the proteins described in (1) and (2),

(b) a process of selecting chemicals binding to the proteins described in (1) and (2),

(8) a screening method for chemicals having a promoting or inhibitory action on the induction of the differentiation of bone cells by the proteins described in (1) and (2), with the method consisting of

(a) a process of bringing test samples into contact with the proteins described in (1) and (2) expressed on the cell surface,

(b) a process of detecting the induction of the differentiation of bone cells by the proteins described in (1) and (2),

(c) a process of selecting the chemicals having a promoting or inhibitory action on the induction of the differentiation of bone cells by the proteins described in (1) and (2), by comparing with the case in which the detection is carried out in the absence of the test samples,

(9) chemicals which can be isolated by the method described in (7) and are capable of binding to proteins as described in (1) and (2),

(10) chemicals which can be isolated by the method described in (8) and are capable of promoting or inhibiting the induction of the differentiation of bone cells by the proteins as described in (1) and (2),

(11) chemicals described in (9) and (10) which are naturally derived,

(12) chemicals described in (9) and (10) which are ligands,

(13) chemicals described in (9) and (10) which are agonists,

(14) chemicals described in (9) and (10) which are antagonists.

The present invention pertains to novel membrane-secreted proteins which are considered to participate in the normal course of bone formation. The base sequence of the mouse-derived cDNA isolated by the present inventors is shown in sequence No.: 3, the amino acid sequence of the signal peptide-containing protein of the proteins encoded by the cDNA is shown in sequence No.: 1, and the amino acid sequence of the mature protein in which the signal peptide at the N-terminal is removed is shown in sequence No.: 2. The isolated clone is named 7F4 by the present inventors. The 7F4 proteins included in the proteins of the present invention have a cysteine-rich region conserved by the TNF receptor subfamily, and the N-terminal (signal sequence region) and the C-terminal possess amino acid regions rich in hydrophobicity. Proteins having amino acid sequences showing significant similarity to that of this 7F4 are not found in the database. Accordingly, the 7F4 proteins are considered novel proteins belonging to the TNF receptor superfamily (Beulter. B, and Huffel. C. V. (1994). Science 264:667-668 Unraveling function in the TNF ligand and receptor families) (Refer to Figure 6).

When the transformed cell KUSA cells (The cells are derived from normal mouse bone marrow stroma cells, and they have been known to have, in addition to hemapoietic indication ability, bone formation stimulating ability by bone marrow induction in vivo transplant. Refer to the reference, Umezawa, A., Maruyama, T., Segawa, K., Shadduck, R.K., Waheed, A., and Hata, J. (1992) Multipotent marrow stroma cell line is able to induce hematopoiesis in vivo. J. Cell. Physiol. 151: 197-205), which had highly expressed 7F4 proteins, was treated with PI-specific phospholipase C, the amount of the proteins on the cell surface was reduced (Application Example 7). Accordingly, it is surmised that the 7F4 proteins do not exist in the intracellular region but have a structure in which they are fixed on the cell surface via a GPI anchor. There are CNTF receptors that are known as GPI anchored membrane proteins. Also, the intracellular region for the IL-6 and IL-11 receptors are very short and do not have a region for signal transduction after ligand binding. These receptors all associate with gp130 after ligand binding, and use the gp130 as the signal transducing strand to transduce the signal into the cells. It is possible that the 7F4, behaving in the same manner as many cytokine receptors do, is also transducing signal into the nuclei by the same mechanism of associating with a signal transducing strand like gp130. In fact, when 7F4 proteins were highly expressed by KUSA cells, the cell morphology changed corresponding to the quantity expressed, and there was a tendency for the cell proliferation rate to decrease (Application Example 6), while the alkaline phosphatase activity, which is an indicator for osteoblast differentiation, increased (Application Example 8). These facts indicate that the 7F4 proteins are specifically related to the differentiation signal for bone cells. Therefore, as shown later, 7F4 proteins and the chemicals binding to them, are considered applicable to preventing and treating bone-related diseases in particular. It is also considered applicable to diagnosis as a bone cell differentiation marker.

It has been reported that, when osteoblasts were continuously cultured over a long period of time, the expression of bone formation marker increased, leading to self-differentiation (Matsumoto T, Igarashi C, Takeuchi Y, Harada S, Kikuchi T, Yamato H, and Ogata E. (1991). Stimulation by 1,25-dihydroxyvitamin D3 of in vitro mineralization induced by osteoblast-like MC3T3-E1 cells. Bone 12:27-32; Sudo H, Kodama HA, Amagai Y, Yamamoto S, and Kasai S. (1983) In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol. 96: 191-198). BMP has been known as the only bone formation factor which induces bone tissue, a factor inducing bone formation through cartilage formation. However, the appearance of cartilage cells are not observed during bone formation by osteoblasts, or by KUSA cells in particular. Therefore, the bone differentiation mechanism cannot be explained by the action of BMP (Wozney, JM, Rosen, V, Celeste, AJ, Mitsock, LM, Whitters, MJ, Kriz, RW, Hewick, RM, and Wang, EA (1988). Novel regulators of bone formation: molecular clones and activities. Science 242:1528-1534), and, at the present time, the

existence of an unknown bone formation factor is considered most likely. 7F4 proteins are powerful candidates for this bone formation factor.

The present invention also pertains to proteins that are functionally equal to 7F4 proteins. As the method for isolating the proteins that are functionally equal to 7F4 proteins, there is the method well-known to those skilled in the art, in which mutations are introduced into the proteins. For example, those skilled in the art are able to prepare proteins that are functionally equal to 7F4 proteins by introducing suitable mutations to the amino acids in 7F4 proteins (sequence No. 1 or 2), using site-directed mutation induction methods (Hashimoto-Gotoh, T, Mizuno, T, Ogasahara Y, and Nakagawa, M. (1985) An oligodeoxyribonucleotide-directed dual amber method for site-directed mutagenesis. Gene 152:271-275; Zoller, MJ, and Smith, M. (1983) Oligonucleotide-directed mutagensis of DNA fragments cloned into M13 vectors, Methods Enzymol. 100:468-500; Kramer, W, Drutsa, V, Jansen, HW, Kramer, B, Pflugfelder, M, and Fritz, HJ (1984). The gapped duplex DNA approach to oligonucleotide-directed mutation construction. Nucleic Acids Res. 12:9441-9456, Kramer W, and Fritz HJ (1987). Oligonucleotide-directed construction of mutations via gapped duplex DNA, Methods Enzymol. 154:350-367; Kunkel, TA (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection, Proc Natl Acad Sci USA 82:488-492). Also, mutation of amino acids occurs in nature. Accordingly, the present invention also includes the proteins that are functionally equal to the 7F4 proteins and having amino acid sequences of which one or a multiple number of amino acids in the amino acid sequences of the 7F4 proteins are mutated. From the functional point of view, the number of mutated amino acids is normally within 30 amino acids, and preferably within 15 amino acids, and more preferably within 5 amino acids, and even more preferably within 3 amino acids.

Additionally, as the other methods well-known to those skilled in the art for the isolation of proteins that are functionally equal, the method which utilizes hybridization may be cited (Sambrook, J et al., Molecular Cloning, 2nd ed., 9.47-9.58, Cold Spring Harbor Laboratory Press, 1989). That is, those skilled in the art could use the DNA sequence (sequence No.: 3) encoding all or part of the 7F4 proteins as the basis and isolate a DNA very similar to it, and further isolate proteins that are functionally equal to 7F4 proteins from the DNA by a normal method. The proteins that are functionally equal to 7F4 proteins and are the proteins encoded by the DNA resulting from hybridization with the DNA or a part of the DNA encoding 7F4 proteins are also the proteins encompassed by the present invention. As examples of these proteins, those mammalian homologues other than from the mouse, for example, may be cited (for example, the proteins encoded by human gene detected by Northern blot in Application Example 3). In general, proteins obtained by hybridization possess amino acid sequences very similar to 7F4

proteins. Very similar generally means a similarity greater than 40% in the amino acid sequence, and preferably a similarity greater than 60%, and more preferably, a similarity greater than 80%.

According to the present invention, "functionally equal" to 7F4 proteins is taken to mean having the same induction activity as 7F4 on the differentiation of bone cells. The induction activity on the differentiation of the bone cells is taken to mean causing the cell proliferation rate for the bone cells to decrease and bringing about morphological changes in the cells. The activity can be detected by, for example, microscopic examination of the bone cell morphology or the determination of the alkaline phosphatase activity, which is generally used as a bone cell differentiation marker (N. C. Partrige, D. Alcorn, V. P. Michelangeli, G. Ryan & T. J. Martin (1983) Cancer Res. 43:4308-14. Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin, J.K. Burns & W. A. Peck (1978) Science 199:542-4. Bone cells: a serum-free medium supports proliferation in primary culture). Determination of the alkaline phosphatase activity may be carried out by, for example, destroying the cells by ultrasound to obtain cell extracts, and incubating with p-nitrophenyl phosphate, which is a substrate for alkaline phosphatase, and quantitatively determining the quantity of p-nitrophenol produced from the decomposition, by the spectrophotometric method. It is also possible to detect osteocalcin or collagen type I as an indicator.

The proteins of the present invention may be prepared as recombinant or natural proteins by methods well-known to those skilled in the art. If they are recombinant proteins, they may be prepared and purified by, for example, expressing and secreting in the cells the part of the proteins of the present invention other than the region required for membrane binding. This is followed by recovering and concentrating the cell culture supernatant, which is then subjected to ion-exchange, reverse-phase or gel filtration chromatography, or to affinity chromatography, by fixing the antibody to the proteins of the present invention to the column, or to a combination of a multiple number of the above columns. The proteins of the present invention may also be prepared and purified by expressing them in a host cell (for example, animal or *E. coli* cells) as fused proteins of the proteins of the present invention and glutathione S-transferase proteins, or as recombinant proteins having multiple histidines added, followed by subjecting the expressed and recombinant proteins to glutathione or nickel columns. Afterward, if necessary, the region in the fused proteins other than the target proteins may be cleaved and removed by methods such as using tropine or factor Xa. If the proteins are the natural ones, they may be prepared, purified and isolated by, for example, using an affinity column to which an antibody of the present invention, which will be described later, is bound, to react with the extract of the cells in which the proteins of the present invention are expressed.

The present invention also pertains to the DNA encoding the aforementioned proteins of the present invention. The DNA of the present invention may be in any forms as long as they can

encode the proteins of the present invention. In other words, it does not matter if they are the cDNA synthesized from mRNA, or from genomic DNA or chemically synthesized DNA. The DNA of the present invention may be prepared by, for example, preparing a cDNA library from the cells in which the proteins of the present invention are expressed, and carrying out hybridization using a part of the DNA sequence of the present invention (for example, the DNA sequence disclosed in sequence No.: 3) as the probe. They may also be prepared by first preparing RNA from the cells in which the proteins of the present invention are expressed and synthesizing an oligoDNA based on the DNA sequence of the present invention (for example, the DNA sequence disclosed in sequence No.: 3), followed by carrying out the PCR reaction using this as a probe and amplifying the cDNA encoding the proteins of the present invention. The DNA of the present invention can be used for the production of the proteins of the present invention as recombinant proteins. Furthermore, when there is a defect in the DNA encoding the proteins of the present invention, its application in the treatment of antisense functional disorders or in gene therapy, by replacement with normal genes, is possible.

The present invention also pertains to vectors in which the DNA of the present invention is inserted. As the vectors of the present invention, for example, when the host cells are *E. coli* cells, there is no particular restriction as long as they possess "ori" for amplification in *E. coli* cells and also possess genes for selection of the transformed *E. coli* cells (for example, drug resistance genes distinguishable by some kind of drug (ampicillin or tetracycline, kanamycin, chloramphenicol)), in order to mass produce the vectors by greatly amplifying them in the *E. coli* cells (for example, JM109, DH5α, HB101, HL1Blue). As the vectors, for example, M13, pUC, pBR322, pBluescript, and pCR-Script may be cited. Additionally, in the cases where cDNA subcloning or splicing is the purpose, pGEM-T, pDIRECT and pT7 may be cited in addition to the above. When a vector is used for the purpose of producing the proteins of the present invention, the expression vectors are particularly useful. As the expression vectors, when the purpose is expression in *E. coli* bacteria, for example, in addition to the aforementioned characteristics of vector amplification by *E. coli* cells, it is essential that they possess promoters (for example, lac, T7, etc.) capable of effective expression in *E. coli* cells, when the host cells are *E. coli* cells such as JM109, DH5α, HB101 or XL1Blue. As such vectors, in addition to the aforementioned vectors, pGEX, pEGFP and pET (in this case, the host is BL21, expressing the T7 RNA polymerase) may be cited.

Additionally, when the purpose is expression in animal cells such as CHO, COS, and NIH3T3 cells, it is essential that they posses promoters (SV40, MMLV-LTR, EF1α, CMV promoters) necessary for expression in the cells, and it is particularly preferable if they possess genes (for example, drug resistance genes distinguishable by drugs (neomycin, G418) for

selecting transformed cells). As the vectors having such characteristics, for example, pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV and pOP13 may be cited.

Furthermore, in the host vector system for the purpose of amplifying the copy number in the cells and in the case of stable production cell lines, a method in which amplification with methotrexate (MTX) is carried out using vectors (for example, pCHOI, etc.) having DHFR genes complementing the CHO cells in which the nucleic acid synthetic pathway is defective, may be cited. Additionally, when the purpose is for temporary expression, a transformation method with vectors (pcD, etc) having the SV40 replicative function using COS cells with the SV40 T antigen on their chromosomes may be cited.

On the other hand, as the methods for the expression of the DNA of the present invention in animals, methods of introducing the DNA of the present invention in combination with a suitable vector into the animals by retroviral, liposomal, cationic liposomal, or adenoviral methods may be cited. There are no specific restrictions on the vectors used, but pAdexcw or pZIPneo, etc. are appropriate. The general genetic operation of insertion of the DNA of the present invention into the vector may be performed by following the conventional method (Molecular Cloning, 5.61-5.63).

The present invention also pertains to host cells in which the vectors of the present invention are introduced. As the host cells in which the vectors of the present invention are introduced, there are no restrictions, and *E. coli* cells or various animal cells may be used. As the *E. coli* cells, for example, JM109, DH5α and HB101may be cited, and, as the animal cells, CHO, COS, 3T3 and HeLa cells may be cited. For the animal cells, when the purpose is high expression, CHO cells are preferred.

On the other hand, there are no specific restrictions the cells used for the expression of the DNA of the present invention, and various animal cells may be cited. However, in the case of gene therapy for bone-related diseases, mesenchymal cells or osteoblasts extracted from the body are particularly preferred as the target cells.

The introduction of vectors into the host cells may be carried out by methods including, for example, the calcium phosphate, DEAE-dextran, electroporation, the lipofection methods, etc.

The present invention also pertains to antibodies binding to the proteins of the present invention. As for the forms of the antibodies of the present invention, there are no specific restrictions, and in addition to polyclonal antibodies, they include monoclonal antibodies. Also, they include antisera, all classes of polyclonal antibodies, monoclonal antibodies obtained from rabbits, etc. immunized with the proteins of the present invention, as well as modified human antibodies or human antibodies from recombinant genes. The antibodies of the present invention may be prepared by the following methods. The polyclonal antibodies may be prepared by, for

example, obtaining serum from small animals such as rabbits immunized with the proteins of the present invention, and using the serum to obtain the fraction recognizing only the proteins of the present invention from an affinity column coupling with the proteins of the present invention, followed by purifying the immunoglobulin G or M from this fraction using a protein A or G column. Also, the monoclonal antibodies are prepared by carrying out immunization of small animals such as the mouse using the proteins of the present invention, and extracting the spleen from the same mouse and mashing it to obtain the cells, followed by fusion with mouse myeloma cells and reagents such as polyethylene glycol, and selecting the clone which produces antibodies to the proteins of the present invention from the hybridoma so obtained. Next, the hybridoma obtained is transplanted intraperitoneally and the peritoneal exudate is recovered, and the monoclonal antibody obtained may be purified by, for example, the ammonium sulfate precipitation method, a protein A column, a protein G column, DEAE ion-exchange chromatography, affinity column coupling with the proteins of the present invention, etc. In addition to being used in the purification and detection of the proteins of the present invention, the antibodies of the present invention can possibly be applied to antibody therapy for bone-related diseases. When they are used for antibody therapy, in order to reduce the immunogenicity, human or human-type antibodies are preferred.

The present invention also pertains to the screening methods for chemicals binding to the proteins of the present invention, using the proteins of the present invention, and it also pertains to the chemicals (for example, ligands, agonists, and antagonists) which are isolated by the screening methods.

One of the forms of the screening methods is that it consists of (a) the process of bringing the test substance into contact with the proteins of the present invention, and (b) the process of selecting chemicals binding to the proteins of the present invention. There are no specific restrictions on the test chemicals which are used in the screening methods of the present invention, and cellular extracts, cell culture supernatants, proteins, peptides, and synthetic low molecular weight substances may be cited as examples. The proteins of the present invention for contact with the chemicals may be brought into contact with the test chemicals as purified proteins, in the form expressed on the cellular membrane, or as the cell membrane fraction. The binding activity of the test chemicals to the proteins of the present invention may be [sic; verb omitted, probably, determined] by the many methods shown later that are well-known to those skilled in the art.

Still another form is that it consists of (a) the process of bringing test chemicals into contact with the proteins of the present invention expressed on the cell membrane, (b) the process of detecting the induction of the differentiation of bone cells by the proteins of the present invention, and (c) the process of selecting chemicals which promote or inhibit the induction of

the differentiation of bone cells by the proteins of the present invention by comparing with the case in which the detection is performed in the absence of the test chemicals. As the cells for the expression of the proteins of the present invention, cells that do not express the ligands which bind to the proteins of the present invention are preferred. The expression of the proteins of the present invention on the cell surface may be carried out by, for example, inserting the DNA encoding the proteins of the present invention into a suitable vector, followed by introducing it into the cells. The induction of the differentiation of the bone cells by the proteins of the present invention may be detected by, for example, morphological examinations of the bone cells microscopically, or methods such as the determination of the of alkaline phosphatase activity, which is generally used as a differentiation marker for bone cells (N. C. Partrige, D. Alcorn, V. P. Michelangeli, G. Ryan & T. J. Martin (1983) Cancer Res. 43:4308-14. Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin, J.K. Burns & W. A. Peck (1978) Science, 199:542-4. Bone cells: a serum-free medium supports proliferation in primary culture). Determination of the alkaline phosphatase activity may be carried out by, for example, destroying the cells to obtain cell extracts, and incubating with p-nitrophenyl phosphate, which is a substrate for alkaline phosphatase, and quantitatively determining the quantity of p-nitrophenol produced from the decomposition, by the spectrophotometric method. It is also possible to detect osteocalcin or collagen type I as an indicator.

Specific methods, for example, the following methods may be used. As the methods using the proteins of the present invention to isolate ligands for the proteins, for example, a method is carried out by preparing the cDNA library from cells (for example, osteoblasts such as KUSA, ROS17/2.8, UMR106-01, UMR106-06, MC3T3E1, HOS-TE85, MG63, SaOS2, UMR206, RCT1 and C3H10T1/2 cells, and fibroblasts such as OP9, stroma and NIH3T3 cells) which are expected to express the ligands using a phage vector (λgt11, ZAP, etc.), expressing it on LB-agarose, and immobilizing the expressed proteins on a filter, followed by preparing the proteins of the present invention as fused proteins with a biotin label or as GST proteins, which are then reacted with the aforementioned filter, and the plaques expressing the binding proteins are detected by streptavidin or anti-GST antibody (western blot method) (Skolnik EY, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A, and Schlessinger J (1991) Cloning of P13 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell 65:83-90). Ligands may also be prepared by fusing the proteins of the present invention with the SRF and GAL4 binding region and expressing it in yeast cells, and preparing a cDNA library that would express the fused form with the VP16 or GAL4 transcription activating region, from cells expected to express the ligands, and introducing it into the aforementioned yeast cells, and isolating the cDNA of library origin from the positive

clones detected and introducing it into *E. coli* bacteria for expression (when the proteins binding to the proteins of the present invention are expressed in yeast cells, the reporter genes are activated due to the binding of the two proteins, and a positive clone can be identified) (two hybrid system) (MATCHMAKER Two-Hybrid System, Mammalian MATCHMAKER Two-Hybrid Assay Kit, MATCHMAKER One-hybrid System) (Each is a product of Clontech Co.), (HybriZAP Two-hybrid Vector System) (Stratagene Co. product), literature (Dalton S, and Treisman R (1992) Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell 68:597-612]). Furthermore, ligands may be prepared by expressing the proteins of the present invention in cells where the ligands are not expressed, and adding to the cells a culture supernatant obtained after introduction of an expression cDNA library which is constructed from a cell estimated to be expressing the ligands, into cells such as COS cells, and searching for the ligands using certain cell changes (proliferation rate, morphology, expression of alkaline phosphatase, etc.), following the "Direct expression cloning method" (Yokota T, Otsuka T, Mosmann T, Banchereau J, DeFrance T, Blanchard D, De Vries JE, Lee F, and Arai K. (1986) Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell- and T-cell-stimulating activities. Proc Natl Acad Sci USA, 83:5894-5898). Furthermore, the ligands may also be prepared by placing a cell culture supernatant of the cell expected to be expressing the ligands of the present invention on an affinity column with the immobilized proteins of the present invention, and purifying the proteins binding specifically to the column. Also, by analyzing the amino acid sequence of the obtained protein (ligand), and synthesizing an oligoDNA based on it and screening the cDNA library using the DNA as a probe, the DNA encoding the ligand may be obtained. In the present invention, the term "ligands" is taken to mean the proteins which bind to the proteins of the present invention expressed on the cell membrane and activate them.

Additionally, as the methods using the proteins of the present invention for isolating agonists or antagonists of the proteins, there are, for example, the method in which chemicals, or a natural product bank, or a random phage dipeptide display library are used to react with the immobilized proteins of the present invention and the molecules which bind are screened, or there is the screening method using combinatorial chemistry techniques (Wrighton NC, Farrell FX, Chang R, Kashyap AK, Barbone FP, Mulcahy LS, Johnson DL, Barrett RW, Jolliffe LK, Dower WJ, Small peptides as potent mimetics of the protein hormone erythropoietin, Science (United States) July 26, 1996, 273:458-64, Verdine GL., The combinatorial chemistry of nature, Nature (England) November 7, 1996, 384:11-13, Hogan JC Jr., Directed combinatorial chemistry, Nature (England), November 7, 1996, 384:17-9), which is the well-known method to those skilled in the art for isolating the agonists or antagonists of the proteins of the present invention. Also, the term "agonists" in the present invention is taken to mean the molecules

which are capable of causing the same phenomenon by binding to the proteins and ligands of the present invention (activating the proteins of the present invention), and capable of binding specifically to the proteins of the present invention. Also, the term "antagonists" is taken to mean the molecules capable of inhibiting the function of the proteins of the present invention by binding to them.

The ligands, agonists and antagonists so isolated may have the following applications. Administering the ligands, agonists, or antagonists, for example, may lead to the induction and activation of the differentiation of osteoblasts, and may increase bone mass and promote bone formation in osteoporosis and osteoarthritis accompanying aging, or may be applied to cancer treatments utilizing the control functions of bone cell differentiation against bone tumors.

Brief description of the figures

Figure 1 shows the cDNA sequence containing the open reading frame of clone 7F4 and the amino acid sequence. The upper section denotes the base sequence and the lower section denotes the amino acid sequence. The line below shows the hydrophobic region, and it is estimated that the N-terminal is the signal sequence while the C-terminal is the region where substitutions with GPI linkers occur.

Figure 2 shows the comparison of the amino acid sequences of 7F4 and the extracellular region of mouse TNFR. The upper sections are the amino acid sequences of the extracellular region of 7F4 and the lower section are those of the mouse TNF receptor. The matching cysteines are shown with an outline font, while the other matching amino acids are shown with lines.

Figure 3 shows the electrophoretogram of the expression of the 7F4 gene in various mouse tissues as analyzed by northern blot.

Figure 4 shows the electrophoretogram of the expression of the 7F4 gene in various human tissues as analyzed by northern blot.

Figure 5A of Figure 5 shows the hydrophobicity of the 7F4 proteins. The left side of the horizontal axis shows the N-terminal of the 7F4 proteins, while the right side shows the C-terminal. Additionally, the vertical axis shows the degree of hydrophobicity. Figure 5B shows the detection of the 7F4 proteins on the surface of KUSA cells and of the 7F4 proteins on the surface of the cells from 2 clones in which 7F4 is highly expressed after treatment with PI-specific phospholipase. The dotted lines in the figure show the results for untreated cells and the solid lines show the results for treated cells.

Figure 6 shows the molecular structure belonging to the TNF receptor superfamily. The repeated sequence rich in cysteine is shown in an oval shape. The horizontal lines in the ovals show the positions of the cysteines.

Figure 7 shows the inhibition of the proliferation of KUSA cells due to high expression of the 7F4 gene. The proliferation rate of the KUSA cells highly expressing the 7F4 gene was determined by tracking the number of the cells over time.

Figure 8 shows the results of detection of the changes in alkaline phosphatase activity due to the expression of the 7F4 gene. The cellular alkaline phospatase activity was determined by using the cell lysate prepared from transformants which highly expressed the 7F4 genes in KUSA cells before they became confluent.

Figure 9 shows the results of detection of the expression of the 7F4 gene in the transformants to which CHO cells and the 7F4 gene were introduced. Each clone obtained from CHO cells transformed by the 7F4 vector was stained with 7F4 antiserum, and the quantity expressed was analyzed by ELITE.

Figure 10 shows the results of detection of the changes in the proliferation rate of COS cell due to high expression of the 7F4 gene. The proliferation rate of the COS cells highly expressing the 7F4 gene was determined by tracking the number of cells over time.

Best embodiment of the present invention

The present invention is further explained by the application examples below, however, they are not to be construed as limiting the present invention.

Application Example 1 Cloning of the 7F4 gene

Cloning of the gene encoding the membrane-secreted proteins expressed in mouse osteoblast KUSA cells was carried out following basically the "signal sequence trap" (SST) method. Specifically, pSRαTac, as the vector for library expression was first constructed by the following method. Plasmid pKCR.Tac-2A, with a human whole-length IL-2 receptor gene inserted (purchased from Riken Gene Bank) was spliced with EcoRI, Eco47III, and a gene fragment encoding the whole length of Tac was obtained. On the other hand, after eliminating the SacI portion inherent to the vector pcD- SRα-FE (Takebe Y, Seiki M, Fujisawa J, Hoy P, Yokota K, Arai K, Yoshita M, Arai N. Mol Cell Biol 8:466-472(1988) SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat) for expression in animal cells, it was spliced with EcoRI. This was then inserted into the aforementioned gene encoding the whole length of Tac to construct pSRαTac (It is possible to remove the signal region of Tac from this plasmid by splicing with EcoRI, SacI). In order to prepare a 5'-enriched cDNA library, first, 5 μg mRNA prepared from KUSA cells was used to synthesize the first strand of the cDNA using random primers. After dC tailing with terminal nucleotidyl transferase at the 5'-terminal, synthesis of the second strand by the Taq DNA

polymerase was carried out using the primer having the EcoRI portion 5'-GCGGCCGCGAATTCTGACTAACTGAC-(dG)17 (sequence No.: 4). This was then broken with ultrasound to make fragments of suitable length, and, after the terminals are blunted, a SacI linker was inserted into the two terminals (CCGCGAGCTCGATATCAAGCTTGTAC (sequence No.: 5) to the 5'-terminal, and GGCGCTCGAGCTATAGTTCGAACATGGAG (sequence No.: 6) to the 3'-terminal. This was made into a template and PCR was carried out from the two primers (5'-GAGGTACAAGCTTGATATCGAGCTCGCGG-3' (sequence No.: 7), 5'-GCCGCGAATTCTGACTAACTGAC-3' (sequence No.: 8) to amplify the cDNA fragment. Subsequently, electrophoresis was performed with a 1.5% agarose gel, and, after a fragment of about 400 bp was sliced from the gel, it was spliced with EcoRI, SacI. This was then inserted at EcoRI and SacI of the expression vector pSRα-TACII prepared by the aforementioned method. Next, this cDNA library was use to transform *E. coli* JM109, and a few pools were prepared, with 49 independent clones in one pool. Plasmids were prepared from each pool and inserted into COS-7 cells using lipofectamine. After two days, they were removed with 0.05% EDTA/PBS, and staining of the cell surface was carried out using mouse anti-IL-2 receptor (Tac) IgG antibody as the primary antibody and FITC-labeled goat anti-mouse IgG antibody as the secondary antibody, and the cells on which Tac proteins were expressed were screened using a flow cytometer (ELITE). The Tac-positive pool was further divided by repeating the above operation until a single clone was obtained. As a result, 3 clones with novel genes having signal sequences were obtained. One of them is a novel gene (entactin2) with high homology to an entactin of a protein constituting the proteins of the base membrane. Another one is a type of membrane proteins that penetrates the membrane 5 to 7 times, and the last one is a novel gene having the repeating cysteine unit conserved by the TNF receptor superfamily (this clone is called 7F4 hereafter). Clone 7F4 contains only a fragment of about 400 bp.

Application Example 2 Cloning of whole length cDNA and determination of the base sequence

In order to isolate a longer fragment, cloning of the 7F4 gene was carried out again from the cDNA library of the KUSA cell with the fragment as the probe, using plaque hybridization. First, the KUSA cDNA library was prepared from the mRNA extracted from the KUSA cells, by oligodT priming using the ZAP-cDNA Synthesis Kit (product of Stratagene Co.), following the accompanying protocol. A phage from about 500,000 clones of this library was spread on a plate and part of the 7F4 cDNA fragment obtained by the SST method was labeled with α^{32}P-dCTP, which was used as the probe to carry out plaque hybridization. After the filter on which the library was immobilized was incubated at 42°C for about 6 h in a hybridization buffer solution (50% formamide, 5X SSPE, 5X Denhardt's solution, 0.1% SDS, 0.1 mg/mL shad sperm DNA), it was further incubated at 42°C for overnight by changing to the same buffer solution containing

the probe. This was then washed a few times with 2X SSC-0.1% SDS and twice with 0.1X SSC-0.1% SDS at 60°C for about 30 min each time, and detection by autoradiography was performed. From the positive clone, splicing was carried out to give plasmid pBluescriptII using ExAssist helper phase (product of Stratagene Co.). The plasmid obtained contained a cDNA fragment of about 3 kb containing an initiating codon. Determination of the base sequence was performed on the base sequence of this cDNA using the primer walking determination method (with the ABI PRISM sequencing kit). When a search for homologues was conducted based on the amino acid sequence estimated from the base sequence determined, no matching gene was found, and it was concluded that the gene encoded by 7F4 is a novel one. (Figure 1). It is very interesting that clone 7F4 has 3 repeating cysteine-rich units commonly conserved in the TNF receptor family, following the signal sequence necessary for membrane expression, and that 7F4 is a novel membrane protein belonging to the TNF receptor superfamily (Figure 2).

Application Example 3 Northern blot

For the mouse, Mouse multiple northern (MTN) blot, and, for humans, human MTN blot, was used for the filter. Hybridization was carried out by labeling the region of the 7F4 cDNA containing the ORF (base sequence numbers 120-480) with $\alpha^{32}P$ using multiprime labeling, and using it as a probe. For the mouse, it was incubated at 68°C for about 2 h using ExpressHyb Hybridization solution (product of CLONTECH CO.), and washed a few times with 2X SSC-0.1% SDS, and twice with 0.1X SSC-0.1% SDS at 60°C. For humans, it was incubated at 68°C for about 4 h using Expresshyb Hybridization solution (product of CLONTECH CO.), and washed twice with 2X SSC-0.05% SDS at 42°C for 10 min. Afterward, it was detected with BAS2000 (product of FUJI Co.). The result showed that, for the mouse, the expression was uneven for each one of the tissue cells (Figure 3). For humans, although uneven expression was also found, the expression in the skeletal muscles and in the heart was particularly high, which was the characteristic aspect (Figure 4). Also, for both the mouse and humans, a band located at about 5kb was detected, and it became clear that the size of the mRNA of this gene was about 5 kb.

Application Example 4 Expression and purification of GST fused proteins

Using plasmid pBluescript-7F4 containing the ORF of the 7F4 cDNA as the template and with 2 primers disclosed in sequence No.: 9 and 10, PCR was performed and the gene encoding the extracellular region of the 7F4 gene was amplified. This was then spliced with BamHI-EcoRI and inserted downstream of the GST gene of pGEX-2T (product of Pharmacia Co.). Induction of GST fused proteins was carried out on *E. coli* JM109 transformed with this plasmid by adding 0.5 mM IPTG, and, after 3 h, the bacteria were collected and suspended in sonication buffer

solution (25 mM Tris pH 8.0, 10 mM EDTA, 1 mM PMSF) containing 1 mg/mL lysozyme. They were broken with ultrasound after standing in ice for 30 min, followed by centrifugation to recover the supernatant. This was then put on the glutathione Sepharose 4G Column (product of Pharmacia Co.) of the Bulk GST purification Module (product of Pharmacia Co.), and the target proteins were eluted and purified following the accompanying protocol. 10% SDS PAGE and staining with Comassie brilliant blue (CBB) were performed. Additionally, it was identified by western blot using anti-GST antibody.

Application Example 5 Preparation of antiserum

The purified 7F4-GST fused protein was used to immunize rabbits after displacement with PBS buffer. The immunization was carried out at 2 week intervals, with the first time at 600 μg/head, and 200 μg/head after the 2nd time. A small sample of blood was drawn after completing the 3rd time, and the specific activity was evaluated by conducting an ELISA. As a result, it was found that the antibody activity increased profoundly. Accordingly, The last immunization was conducted and whole blood sampling was carried out by drawing blood from the heart.

Application Example 6 Establishing the 7F4 gene expression cell line

PCR amplification was carried out on the gene containing the entire ORF of 7F4 with the two primers disclosed in sequence No.: 11 and 12, which was then spliced with EcoRI-BamHI. This gene fragment was inserted downstream of the EF1α promoter of the expression vector pCOSI having the neomycin gene as a drug selection marker (Sato K, Tsuchiya M, Saldanha J, Koishihara Y, Ohsugi Y, Kishimoto T, Bendig MM, (1994), Mol Immunol. 31:371-381, Humanization of a mouse anti-human interleukin-6 receptor antibody comparing two methods for selecting human framework regions), and pCOSI-7F4 was prepared. After the plasmid (25 μg) was spliced with PvuI and inserted into KUSA cells (7 x 10^6 cells) by electroporation (1.6 kV, 25 μF, time constant 0.36), incubation was carried out for several days using a medium (IMDM + 10% FCS) containing 480 μg/mL G418, and more than 10 clones so produced were selected. After the cell surfaces of these clones were stained with antiserum ([diluted] x 1000) and FITC-labeled anti-rabbit IgG (H + L), analysis by flow cytometer ELITE (product of COULTER Co.) was carried out, and a few cells highly expressing the 7F4 gene were selected by comparing with the parent KUSA cells.

Application Example 7 Detection of change of expression of 7F4 proteins on the cell surface by treatment with phosphotidyl inositol specific phospholipase C

When the hydrophobicity of the amino acid sequence of 7F4 was analyzed using hydrophobicity analysis software (DNASIS Co.), it was found that, in addition to the N-terminal, which was the region encoding the signal sequence, a region rich in hydrophobicity also existed at the C-terminal, and that the ORF ended at that region (Figure 5A). From the molecular structure of the amino acids, it is estimated that there is a possibility that the molecules do not possess an intracellular region and that the proteins are binding to the cellular membrane by the glycosyl-phosphotidyl inositol (GPI) anchor (Ikezawa H, Yamanegi M, Taguchi R, Miyashita T, and Ohyabu T (1976) Studies on phosphatidylinositol phosphodiesterase (phospholipase C type) of *Bacillus cereus*. I. Purification, properties and phosphatase-releasing activity. Biochim Biophys Acta. 450:154-164, Low MG, and Finean, JB (1977) Release of alkalinene phosphatase from membranes by a phosphotidylinositol-specific phospholipase C. Biochem. 167:281-284, Low MG, and Saltiel AR (1988), Structural and functional roles of glycosyl-phosphatidylinositol in membranes, Science 239:268-275). At this point, KUSA cells or the cell lines obtained which highly express the 7F4 gene in KUSA cells (KUSA-7F4 #2; low expression, [KUSA-7F4 #5: high expression) were established, and treatment with phosphotidyl inositol-specific phospholipase C was carried out, and the quantitative change of the 7F4 proteins on the cellular surface was analyzed by a flow cytometer (ELITE) after staining with antiserum. After washing with PBS, the cells were incubated in buffers (PBS + 1% FCS) with and without phosphotidylinositol-specific phospholipase C (2 U/mL; product of Funakoshi Co.) for 1 h at 37°C. The result revealed that, when treating with phosphotidylinositol-specific phospholipase C, the number of cells positive for 7F4 proteins on the cellular surface decreased for each of the cell lines. (Figure 5B). From this result, it became clear that the 7F4 molecules had a structure that could be cleaved on the cellular surface by phosphotidylinositol-specific phospholipase C. It also became clear from this result that there is a possibility that the 7F4 gene is expressed on the cellular surface by binding with a GPI anchor, that is , it is a GPI-type membrane protein.

Application Example 8

Detection of the proliferation and differentiation of cells by high expression of 7F4 proteins, and detection of change in the alkaline phosphatase activity

(1) Establishing 7F4 gene expression cell line

The expression vector pCOSI-7F4 (25 μg) for the 7F4 gene, controlled by the EF1α promoter, was spliced with PvuI and introduced into KUSA (7 x 10^6 cells) or CHO cells by electroporation (1.6 kV, 25 μF, time constant 0.36), which were incubated for several days in a

culture medium (IMDM + 10% FCS or α-MEM + 10% FCS) containing 480 μg/mL G418, and more than 10 grown clones were selected. After the cellular surfaces of these clones were stained with 7F4 antiserum (x1000) and FITC-labeled antirabbit IgG (H + L), flow cytometer (ELITE) analysis was performed, and those having high 7F4 gene expression when compared to the parent KUSA and CHO cells were selected. From this, transformed cell line #2 weakly expressing 7F4 proteins and the transformed cell line #11 highly expressing 7F4 proteins, in which pCOSI-7F4 was introduced, were obtained. When incubation of these transformants was continued, the morphological changes in the cells were significant. The parent KUSA cells showed the long and thin fibroblast form, while all the transformants spread largely and widely with an increase in tiny protuberances.

(2) Proliferation analysis

Incubations were carried out by applying 5 x 10^3 cells/well (KUSA cells) and 1 x 10^3 cells/well (CHO cells) in 12-well plates, and the number of cells were counted by removing the cells over time. The expression level of the exogenous 7F4 in the transformed cells was the highest for #11, which was followed by #8 and #5 at a medium degree, and #2 was the lowest. Corresponding to the lower amounts of exogenous 7F4 expressed, the proliferation rate of these cells was lower compared to the parent cells (Figure 6). From these results, it was found that the high expression of the 7F4 gene could further induce the transformation of the osteoblasts.

(3) Assay of alkaline phosphatase activity

After washing the cells incubated in 6 wells with PBS twice, they were removed using 700 μL of sonication buffer solution (50 mM Tris pH 7.2, 0.1% Triton X-100). These were then gently broken by ultrasound and centrifuged at 15000 rpm for 15 min. The protein concentration was then determined using a protein assay kit (product of Bio-Rad). To 2-20 μg of the cellular substances, an incubation buffer solution (0.1M 2-amino-2-methyl-1-propanol HCl, pH 10.5, 2 mM $MgCl_2$) containing an equal amount of 20 mM p-nitrophenol phosphate substrate were added, which was incubated for 30 min at 37°C. After terminating the reaction by adding NaOH to 0.1N, a colorimetric determination was carried out at OD_{405} of the amount of p-ntrophenol formed. Parent KUSA cells and transformed cell lines #2 and #11 were cultured, and when the cells became about 80% confluent, that is, when they were in the proliferation period, cell lysates were prepared from the cells, and the activity of the intracellular alkaline phosphatase, which is one of the differentiation markers for osteoblasts, was determined. As a result, it was found that the activity of the alkaline phosphatase also increased in correspondence with the expression of the exogenous 7F4 (Figure 7).

The same experiment was performed with CHO cells. Similarly to the KUSA cells, pCOSI-7F4 was introduced into CHO cells, and several cell lines highly expressing exogenous 7F4 were established (Figure 8). An analysis was then carried out to assess how the proliferation of these transformed cells was affected by the high expression of 7F4. Contrary to expectation, in the CHO cells, the cell proliferation was not affected by the high expression of 7F4 at all (Figure 9). Neither was there any change in the cell morphology.

Applications possibility in the industrial field

The present invention provides novel membrane-secreted proteins belonging to the TNF receptor superfamily and believed to be participating in the differentiation of the osteoblasts, and genes encoding the proteins, vectors into which the genes are inserted, the host cells sustaining the vectors, and antibodies to the proteins. It also provides a screening method using the proteins for prospective chemical candidates for drug preparations. The chemicals isolated using the proteins, genes, antibodies and screening method of the present invention may be utilized as pharmaceuticals. It has been expected that bone-related diseases, beginning with osteoporosis, would increase accompanying the aging of population. The proteins of the present invention are thought to participate in the differentiation and activation of osteoblasts, which are important in the course of bone formation, and the proteins of the present invention, their antibodies and ligands, are considered capable of making a contribution to the treatment of bone diseases. Also, they are considered capable of making, a contribution to the elucidation of the mechanism of bone formation.

Sequence tables

(1) Name(s) of applicant(s): Chugai Research Institute for Molecular Medicine Co., Ltd.

(2) Name of invention: Novel membrane-secreted proteins

(3) File No.: C1-806PCT

(4) Application No.:

(5) Date of Application:

(6) Name of country and application number of which the application becomes the basis of priority: Japan; Hei 9 [1997]-099653

(7): Priority date: April 1, 1997

(8) Number of sequences: 12

Sequence No.: 1

Length of sequence: 176

Topology: Straight chain

Type of sequence: Protein

Sequence

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Trp Phe Leu Leu Leu Leu Leu Leu Asn Leu Phe Leu Pro Val Ile Phe
    -15                 -10                 -5
Ala Met Pro Glu Ser Tyr Ser Phe Asn Cys Pro Asp Gly Glu Tyr Gln
 1              5                  10                  15
Ser Asn Asp Val Cys Cys Lys Thr Cys Pro Ser Gly Thr Phe Val Lys
            20                  25                  30
Ala Pro Cys Lys Ile Pro His Thr Gln Gly Gln Cys Glu Lys Cys His
        35                  40                  45

Pro Gly Thr Phe Thr Gly Lys Asp Asn Gly Leu His Asp Cys Glu Leu
    50                  55                  60
Cys Ser Thr Cys Asp Lys Asp Gln Asn Met Val Ala Asp Cys Ser Ala
65                  70                  75                  80
Thr Ser Asp Arg Lys Cys Glu Cys Gln Ile Gly Leu Tyr Tyr Tyr Asp
                85                  90                  95
Pro Lys Phe Pro Glu Ser Cys Arg Pro Cys Thr Lys Cys Pro Gln Gly
            100                 105                 110
Ile Pro Val Leu Gln Glu Cys Asn Ser Thr Ala Asn Thr Val Cys Ser
        115                 120                 125
Ser Ser Val Ser Asn Pro Arg Asn Trp Leu Phe Leu Leu Met Leu Ile
    130                 135                 140
Val Phe Cys Ile
145
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Sequence No.: 2
Length of sequence: 148
Topology: Straight chain
Type of sequence: Protein
Sequence

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 1               5                  10                  15
Ser Asn Asp Val Cys Cys Lys Thr Cys Pro Ser Gly Thr Phe Val Lys
            20                  25                  30
Ala Pro Cys Lys Ile Pro His Thr Gln Gly Gln Cys Glu Lys Cys His
        35                  40                  45
Pro Gly Thr Phe Thr Gly Lys Asp Asn Gly Leu His Asp Cys Glu Leu
    50                  55                  60
Cys Ser Thr Cys Asp Lys Asp Gln Asn Met Val Ala Asp Cys Ser Ala
65                  70                  75                  80
Thr Ser Asp Arg Lys Cys Glu Cys Gln Ile Gly Leu Tyr Tyr Tyr Asp
                85                  90                  95
Pro Lys Phe Pro Glu Ser Cys Arg Pro Cys Thr Lys Cys Pro Gln Gly
            100                 105                 110
Ile Pro Val Leu Gln Glu Cys Asn Ser Thr Ala Asn Thr Val Cys Ser
        115                 120                 125
Ser Ser Val Ser Asn Pro Arg Asn Trp Leu Phe Leu Leu Met Leu Ile
    130                 135                 140
Val Phe Cys Ile
145
```

Sequence No.: 3
Length of sequence: 1509
Number of strand: Double-strand
Topology: Straight chain
Type of sequence: cDNA to mRNA
Characteristics of Sequence

Symbol denoting characteristics: CDS
Existing location: 12 ..542
Method of determining the characteristics: E

Symbol denoting characteristics: sig peptide
Existing location: 12 ..95
Method of determining the characteristics: S

Symbol denoting characteristics: mat peptide
Existing location: 96 ..542
Method of determining the characteristics: S

Sequence

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             -28         -25                 -20

TGG TTC CTC TTG CTG CTG CTG CTG AAT CTG TTC TTG CCG GTA ATA TTT        95
Trp Phe Leu Leu Leu Leu Leu Leu Asn Leu Phe Leu Pro Val Ile Phe
    -15                 -10                 -5

GCT ATG CCT GAA TCA TAC TCC TTC AAC TGT CCC GAT GGT GAA TAC CAG       143
Ala Met Pro Glu Ser Tyr Ser Phe Asn Cys Pro Asp Gly Glu Tyr Gln
  1               5                  10                  15

TCT AAT GAT GTC TGT TGC AAG ACC TGT CCC TCA GGT ACA TTT GTC AAG       191
Ser Asn Asp Val Cys Cys Lys Thr Cys Pro Ser Gly Thr Phe Val Lys
            20                  25                  30

GCG CCC TGC AAA ATC CCC CAT ACT CAA GGA CAA TGT GAG AAG TGT CAC       239
Ala Pro Cys Lys Ile Pro His Thr Gln Gly Gln Cys Glu Lys Cys His
        35                  40                  45

CCA GGA ACA TTC ACA GGG AAA GAT AAT GGC CTG CAT GAT TGT GAA CTT       287
Pro Gly Thr Phe Thr Gly Lys Asp Asn Gly Leu His Asp Cys Glu Leu
    50                  55                  60
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TGC TCC ACC TGT GAT AAA GAC CAG AAT ATG GTG GCT GAC TGT TCT GCC      335
Cys Ser Thr Cys Asp Lys Asp Gln Asn Met Val Ala Asp Cys Ser Ala
 65                  70                  75                  80
ACC AGT GAC CGG AAA TGC GAG TGC CAA ATA GGT CTT TAC TAC TAT GAC      383
Thr Ser Asp Arg Lys Cys Glu Cys Gln Ile Gly Leu Tyr Tyr Tyr Asp
                 85                  90                  95
CCA AAA TTT CCG GAA TCA TGC CGC CCA TGT ACC AAG TGT CCC CAA GGA      431
Pro Lys Phe Pro Glu Ser Cys Arg Pro Cys Thr Lys Cys Pro Gln Gly
            100                 105                 110
ATC CCT GTC CTC CAG GAA TGC AAC TCC ACA GCT AAC ACT GTG TGC AGT      479
Ile Pro Val Leu Gln Glu Cys Asn Ser Thr Ala Asn Thr Val Cys Ser
        115                 120                 125
TCA TCT GTT TCA AAT CCC AGA AAC TGG CTG TTC CTA CTG ATG CTA ATT      527
Ser Ser Val Ser Asn Pro Arg Asn Trp Leu Phe Leu Leu Met Leu Ile
    130                 135                 140
GTC TTC TGT ATC TGAAGAAGAT AAAGGTTCTA CAGATGGTGT CTGTAGCTTC          579
Val Phe Cys Ile
145
CTTTTATTGC TGTGAAGAGA AACCATGGAG GCAACTCTTT CATTTTATTT TATTTTTTAA     639
TGTCTTGAAC TTGATTTGAA GACCAGGCTG GACTCAAACT CACAGAGATC CGGACTAGGC     699
ACCTCTAATA TAGGAAAACA TTGAATTGGG ACTGGCTTAC AGTTTCAGAA GTTCTGTCCA     759
TGATTATCAT AGTGCGAAGC ATGGAGGCAC GGAGGCACAC ATGGTGCTGG AGAAGAAGCT     819
GAGAGTTCTG CATCTTGATC TGCAAGCAAT AAAAGGAGAC TGTGTGCCAC ACTACACATA     879
GCTTGAACAT AGGAGACCTC AAAGCCTGTC CCCACAGTGA CAAACTTCCT CCAACAAGGT     939
CATACCTCCT AATAATACCA TTTCTTATGA GGCAAGCATT CAAACACATG AGTCTATGAG     999
GGCCAAACCA ATTCAAACCA CCACAGGTTA ACAATTGCCC TCTGCAGCTC TCTGGTGGAG    1059
```

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GAAACTCAGG ATGAATGGTC CACTGTGGTT CCTATTAACA TACTGAAGAA CATGACCTCA 1179

CCTTAGACTT CTCCACCTCA CTGGCTTCCC TTCCCCTAGC TTCTCATTCC CAGGTAACCC 1239

TGCCATTTTT TGGTAATGTG CCTTCTTGGT TCTTCCTCTC CTTTCCCCCT CTCTTCTGGT 1299

CCTTATTTCT CTTCCTCTCC CACTCTCCAC CAGCCGCCTC TTAAGGCCTG AGTCAGTCTG 1359

CAGGCCATGT TTAATCTACT ACTTTCTCTC TGCTCTGGAC TCATCCAGAT GTCTCTGGCT 1419

GAGCTCTCCC TCCTATCTAC AATAAAACCT TCCCCCTAAC CAGAAATGGA ACAGTTTTGT 1479

CCTCACTTTG TACATCTGGT GCCTGAAACC 1509

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Length of sequence: 43
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA
Sequence
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Sequence No.: 5
Length of sequence: 26
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA
Sequence
CCGCGAGCTC GATATCAAGC TTGTAC 26

Sequence No.: 6
Length of sequence: 29
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA
Sequence
GGCGCTCGAG CTATAGTTCG AACATGGAG 29

Sequence No.: 7

Length of sequence: 29
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA
Sequence
GAGGTACAAG CTTGATATCG AGCTCGCGG 29

Sequence No.: 8
Length of sequence: 23
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA
Sequence
GCCGCGAATT CTGACTAACT GAC 23

Sequence No.: 9
Length of sequence: 24
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA
Sequence
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Sequence No.: 10
Length of sequence: 26
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA
Sequence
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Sequence No.: 11
Length of sequence: 36
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA

Sequence
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Sequence No.: 12
Length of sequence: 35
Number of strand: single strand
Topology: Straight chain
Type of sequence: Other nucleic acid, synthetic DNA
Sequence
CCGGATCCTC AGATACAGAA GACAATTAGC ATCAG 35

Claims

1. Proteins formed by the amino acid sequence shown in sequence No.: 1 or 2, and those having the amino acid sequences resulting from substitutions, deletions or additions of one or more amino acids in the amino acid sequences of the proteins, and these proteins having induction activity on the differentiation of bone cells,

2. Proteins encoded by DNA hybridizing with the DNA having the base sequence of sequence No.: 3, and the proteins having induction activity on the differentiation of bone cells,

3. DNAs encoding the proteins described in (1) and (2),

4. Vectors inserted with the DNA described in Claim 3,

5. Host cells sustaining the vectors described in Claim 5,

6. Antibodies binding to the proteins described in (1) and (2),

7. A screening method for chemicals binding to the proteins described in (1) and (2), with the method consisting of

(a) a process of bringing test samples into contact with the proteins described in (1) and (2),

(b) a process of selecting chemicals binding to the proteins described in (1) and (2),

8. A screening method for chemicals having a promoting or inhibitory action on the induction of the differentiation of bone cells by the proteins described in (1) and (2) on the differentiation of bone cells, with the method consisting of

(a) a process of bringing test samples into contact with the proteins described in (1) and (2) expressed on the cell surface,

(b) a process detecting the induction of the differentiation of bone cells by the proteins described in (1) and (2)

(c) a process of selecting the chemicals having a promoting or inhibitory action on the induction of the differentiation of bone cells by the proteins described in (1) and (2) by comparing with the case in which the detection is carried out in the absence of the test samples,

9. Chemicals which can be isolated by the method described in Claime 7 and are capable of binding to proteins as described in (1) and (2),

10. Chemicals which can be isolated by the method described in Claim 8 and are capable of promoting or inhibiting the induction of the differentiation of bone cells by the proteins as described in (1) and (2)

11. Chemicals described in (9) and (10) which are naturally derived,

12. Chemicals described in (9) and (10) which are ligands,

13. Chemicals described in (9) and (10) which are agonists,

14. Chemicals described in (9) and (10) which are antagonists.

```
   1 AGCTCACAGCC                                                          11
  12 ATGGTTACCTTCAGCCACGTCTCCAGTCTGAGTCACTGGTTCCTCTTGCTGCTGCTGCTG        71
   1 M  V  T  F  S  H  V  S  S  L  S  H  W  F  L  L  L  L  L  L         20
  72 AATCTGTTCTTGCCGGTAATATTTGCTATGCCTGAATCATACTCCTTCAACTGTCCCGAT       131
  21 N  L  F  L  P  V  I  F  A  M  P  E  S  Y  S  F  N  C  P  D         40
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  41 G  E  Y  Q  S  N  D  V  C  C  K  T  C  P  S  G  T  F  V  K         60
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  61 A  P  C  K  I  P  H  T  Q  G  Q  C  E  K  C  H  P  G  T  F         80
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 312 AATATGGTGGCTGACTGTTCTGCCACCAGTGACCGGAAATGCGAGTGCCAAATAGGTCTT       371
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 121 Y  Y  Y  D  P  K  F  P  E  S  C  R  P  C  T  K  C  P  Q  G        140
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 141 I  P  V  L  Q  E  C  N  S  T  A  N  T  V  C  S  S  S  V  S        160
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 161 N  P  R  N  W  L  F  L  L  M  L  I  V  F  C  I  *                 177
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 961 TTCTTATGAGGCAAGCATTCAAACACATGAGTCTATGAGGGCCAAACCAATTCAAACCAC      1020
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1501 CCTGAAACC                                                         1509
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Figure 1

```
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mTNFR  CPGGKYVHSKNNSIC CTKCHKGTYLVSDCP SPGRDTVCRECEKGT FTASQNYLRQCLSCK 60

7F4    TCDKD--QNMVADCS ATSDRKCEC---QIG LYYYDPKFPESCRPC TKCPQGIPVLQECNS 120
mTNFR  TCRKEMSQVEISPCQ ADKDTVCGCKENQFQ RYLSETHFQ--CVDC SPCFNGTVTIP-CKE 120

7F4    TANTVC                                                          126
mTNFR  TQNTVC                                                          126
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Figure 2

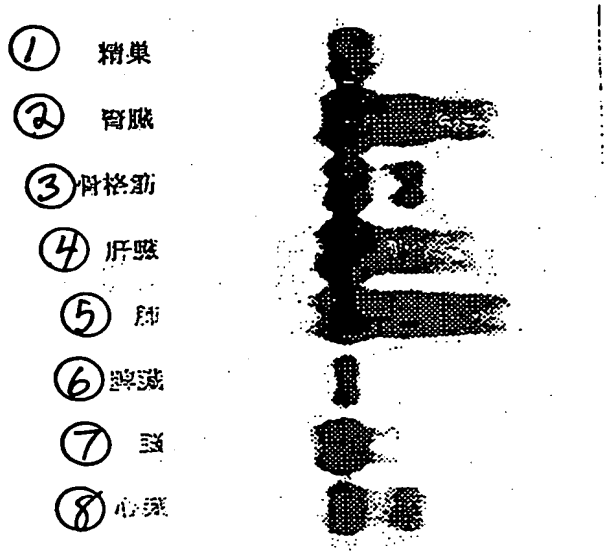


Figure 3

Key:
1 Testis
2 Kidney
3 Skeletal muscle
4 Liver
5 Lung
6 Spleen
7 Brain
8 Heart

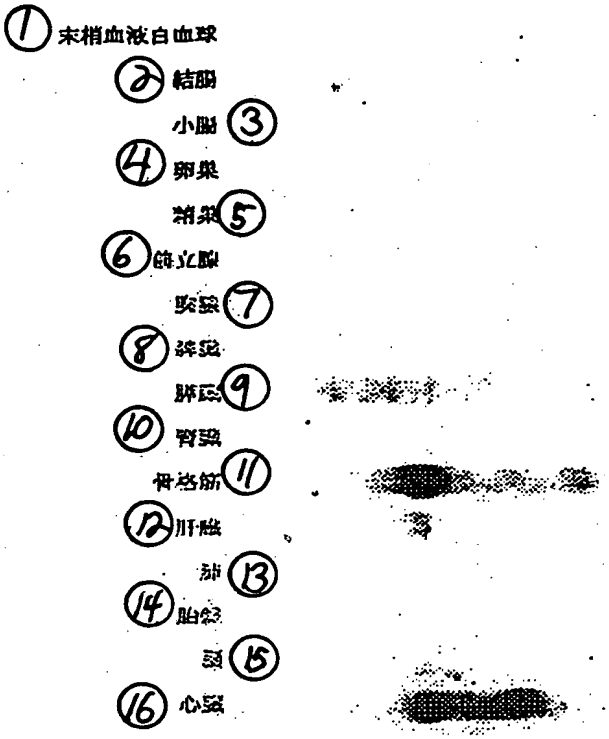
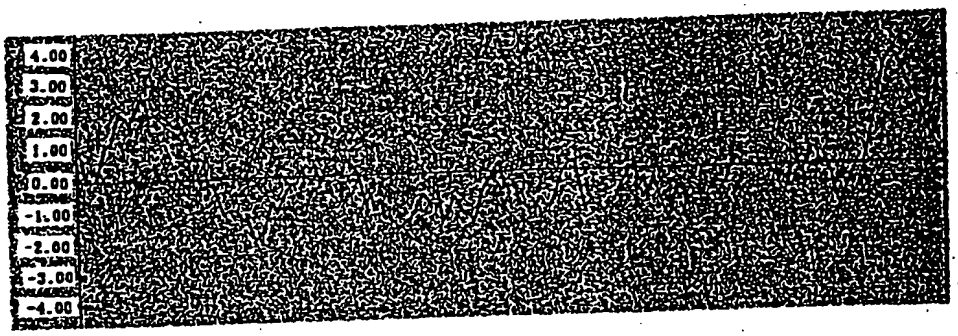


Figure 4

Key:

1	Peripheral blood white blood cells
2	Colon
3	Small intestine
4	Ovary
5	Testis
6	Prostate
7	Thymus
8	Spleen
9	Pancreas
10	Kidney
11	Skeletal muscle
12	Liver
13	Lung
14	Placenta
15	Brain
16	Heart

A.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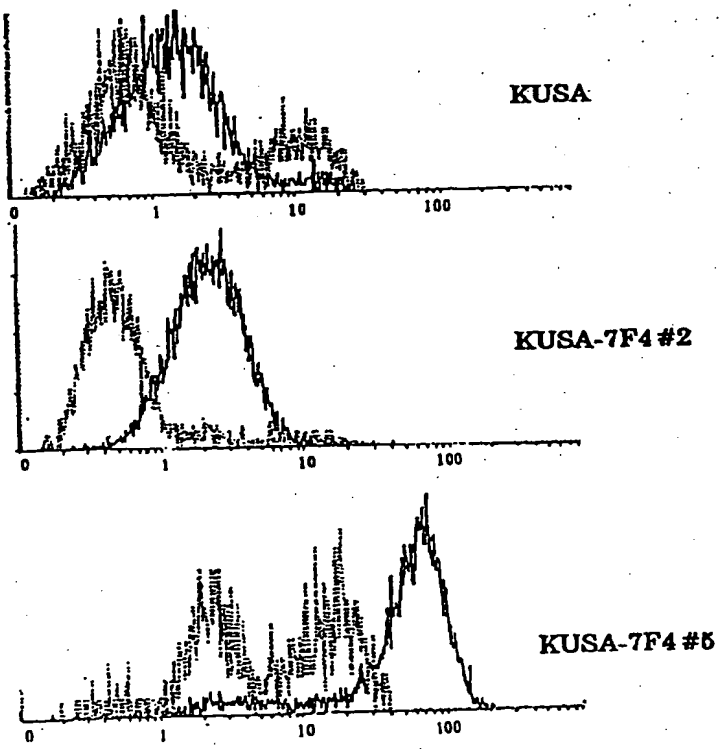
B.


KUSA
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100
KUSA-7F4 #2
0
1
10
100
KUSA-7F4 #5
0
1
10
100

Figure 5

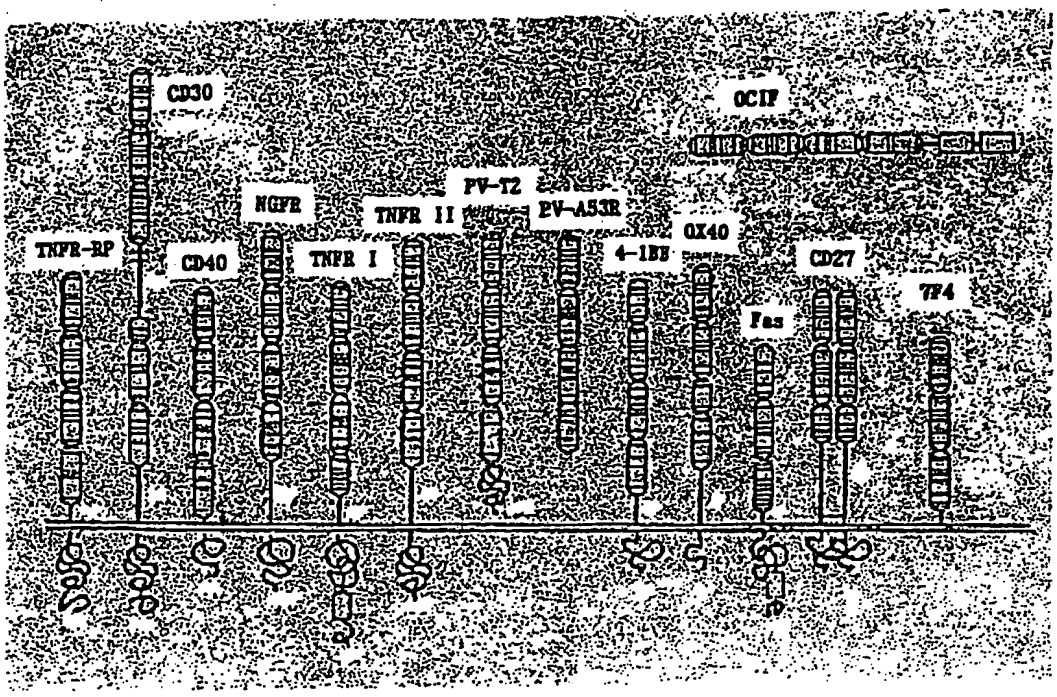

Figure 6

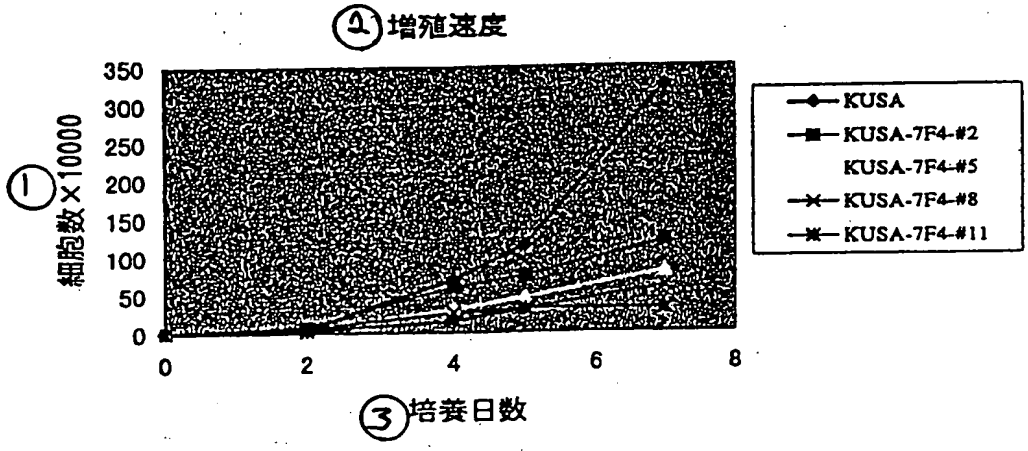

Figure 7

Key: 1 Number of cells
2 Proliferation rate
3 Days of incubation

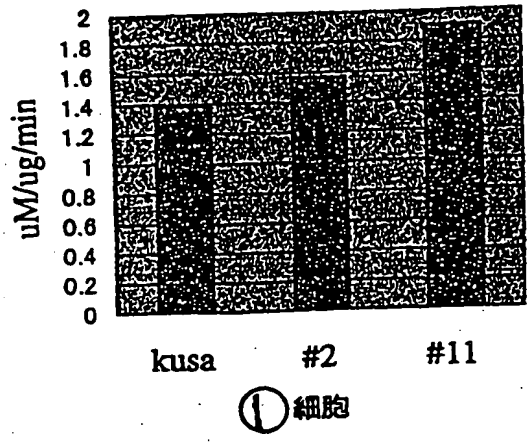

Figure 8

Key: 1 Cell

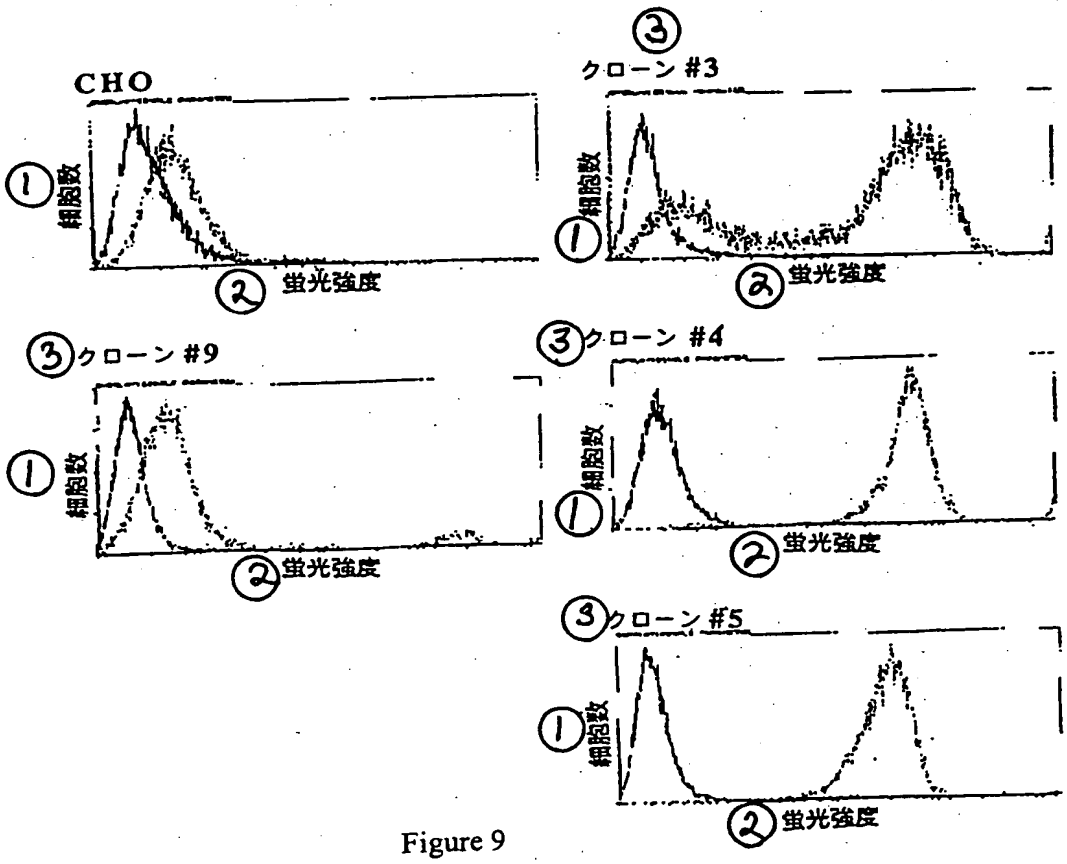

Figure 9

Key: 1 Number of cells
2 Fluorescent intensity
3 Clone

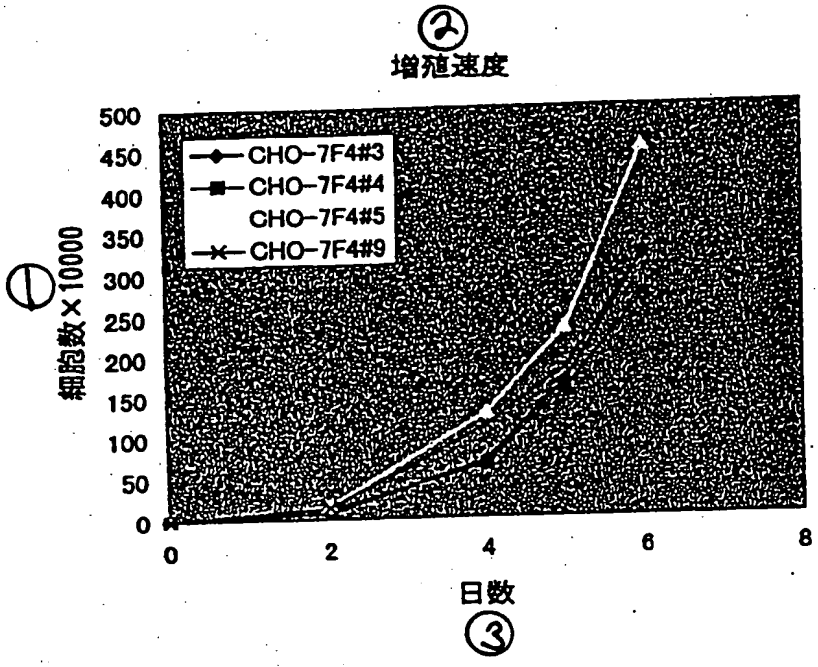

Figure 10

Key: 1 Number of cells
2 Proliferation rate
3 Days

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/01511

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C07K14/51, C12N15/12, C12N5/10, C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C07K14/51, C12N15/12, C12N5/10, C07K16/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DDBJ/GenBank/EMBL, GENESEQ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 7-118296, A (Sumitomo Metal Industries, Ltd.), May 9, 1995 (09. 05. 95) (Family: none)	1-14
A	JP, 9-31098, A (Hoechst Japan Ltd.), February 4, 1997 (04. 02. 97) (& WO, 97/04095, A)	1-14
A	JP, 8-510446, A (Creative Biomolecules, Inc.), November 5, 1996 (05. 11. 96) (& WO, 94/20539, A EP, 687272, A)	1-14
A	JP, 9-501305, A (Genetics Institute, Inc.), February 10, 1997 (10. 02. 97) (& WO, 94/26893, A EP, 698095, A)	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
June 18, 1998 (18. 06. 98)	June 30, 1998 (30. 06. 98)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)